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THE EFFECT OF THYMIDINE POOL SIZE ON THE INCORPORATION OF THYMIDINE INTO DNA AFTER IRRADIATION

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FOREWORD

This report was prepared in the Radiobiology Branch under task No. 775702. It was submitted for publication on 27 August 1965. The work was accomplished between May 1964 and May 1965.

The experiments reported herein were conducted according to the "Principles of Laboratory Animal Care" established by the National Society for Medical Research.

This report has been reviewed and is approved.

Harold V. Ellingson

HAROLD V. ELLINGSON
Colonel, USAF, MC
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ABSTRACT

A model system to explain the effect of irradiation on the incorporation of thymidine into deoxyribonucleic acid (DNA) was studied. The model explains the increase observed in thymidine incorporation after irradiation as due to an increase in the intracellular thymidine pool, coming from DNA breakdown. The data agree with this model; however, further research on this concept is necessary.

THE EFFECT OF THYMIDINE POOL SIZE ON THE INCORPORATION OF THYMIDINE INTO DNA AFTER IRRADIATION

I. INTRODUCTION

The incorporation of isotopically labeled thymidine into deoxyribonucleic acid (DNA) has been used by many investigators to study the effect of irradiation on the rate of DNA synthesis. These investigators (1, 2, 3, 5) have assumed that the endogenous thymidine triphosphate pool is not affected significantly by either the added tracer compound and unlabeled carrier or by the irradiation. This assumption has been questioned by many workers in this field (1, 2), and limited experiments have been interpreted to show that it is valid. Recently, however, Stewart et al. (4) have raised serious objections to the use of this technic in irradiated animals on the grounds that the assumption of a constant thymidine pool size is not valid. They formulated a mathematical model for DNA synthesis in tissues and have presented experimental data to support this model. The model can be used to predict that the change observed in DNA synthesis after irradiation is due to an increase in the size of the thymidine triphosphate pool. Experimental data to support this prediction are also presented.

Using a previously described system for studying the incorporation of thymidine 2-C^{14} into DNA in vitro (5) as well as the system described by Nygaard and Potter (1) in vivo, we have studied the effect of pool size on the incorporation of thymidine into the DNA of rat spleens over a wide range of pool sizes. In general, our data support the concept of Stewart et al. (4).

II. MATERIALS AND METHODS

Irradiation

The irradiations were carried out in a ventilated Plexiglas box in the USAF School of Aerospace Medicine multikilocurie Co^{60} facility and were done under conditions of maximal backscatter (6). A dose of 975 rads was given in 4 minutes. The exposure dose rate was measured with a Victoreen R meter. The absorbed dose rate was calculated by adjusting the exposure dose rate by 0.975.

Incubation medium for in vitro studies

The medium used for the incubation of tissues was Krebs-Ringer phosphate (7) modified to contain 10 μM. of glucose per milliliter and with the calcium eliminated.

Preparation of thymidine

Thymidine 2-C^{14} (specific activity, 30 mc./mM.) and thymidine-methyl-H^3 (specific activity, 6.7 c./mM.) were obtained from New England Nuclear and diluted with physiologic saline solution before use.

Method

Male Sprague-Dawley rats, weighing 250 to 350 gm., were used for all studies. For the in vivo studies, a dose of 4.5 μc. of thymidine 2-C^{14} was injected intraperitoneally into sham-irradiated control rats and into rats which had received 975 rads 30 minutes previously. Exactly 30 minutes later, the rats were sacrificed

by decapitation, and the spleens were excised and quick-frozen in a Dry Ice–acetone mixture. An identical study was done on a second group of animals, which were irradiated with 975 rads and injected with 100 μc. of thymidine-methyl-H^3.

For the in vitro studies, rats were sacrificed by decapitation 30 minutes after irradiation, and the spleens were removed and sliced on a cold block. The slices were suspended in 4 ml. of modified KRP. Sham-irradiated controls were treated in a similar manner. The resulting suspensions were incubated with gentle shaking in Erlenmeyer flasks in a constant-temperature water bath at 37.5° C. At the start of incubation, 0.25 μc. of thymidine 2-C^{14} was added to the medium, the flasks were flushed with 100% 0_2 for 10 minutes and sealed with rubber stoppers, and the incubation continued for a total of 30 minutes. To stop the reaction, the flasks were chilled in an ice bath.

Before the DNA extraction was performed, the frozen spleens from the in vivo experiment were thawed, approximately 200 mg. samples were sliced on a cold block, and the slices suspended in 4 ml. of cold 0.9% saline.

To extract the DNA, the cell suspensions, either from the in vitro or in vivo studies, were homogenized in 9 ml. of 0.3 *M* sucrose at 2° C. and then centrifuged at 2° C. at 600 × G. The residue was washed 3 times with ice cold 0.3 *N* perchloric acid, once with absolute alconol, then twice with a 3-1 ethanol-ether mixture. Two ml. of 5% trichloracetic acid were added, and the residue was then heated for 15 minutes at exactly 90° C. Portions of the supernatant were then taken for liquid scintillation counting and DNA determination by the Burton modification of the diphenylamine method (8).

The samples were counted in a model 720 Nuclear Chicago automatic scintillation counter with the use of a dioxane-alcohol counting system (9). The counts were corrected for quenching due to water, trichloracetic acid, and hydrolysis products by use of the internal standard method. This quenching varied from 15 to 25%. Counting efficiency for carbon 14 was 35% and for tritium was 3%.

Data presentation

In order to make possible a comparison of our data and the data of Stewart et al., the results are presented by using the parameters which they chose. The pool size and the incorporation of thymidine are expressed as molecules of thymidine per cell. To calculate these numbers, a variety of assumptions had to be made. It is assumed that the thymidine is uniformly distributed in the rat in vivo and that there is no barrier between the intracellular and extracellular pools. We have used a value of 10^9 cells per gram of tissue and have also used a value of 1.5 mg. of DNA per 100 mg. of spleen tissue. These data have been experimentally obtained in our laboratory and agree with the published data (3, 10). Table I shows the range of pool sizes used and the assay systems chosen for study.

TABLE I

Pool sizes and assay systems

Assay system	Isotope	Specific activity (mc./mM.)	Pool size (molecule/cell)		
			Labeled	Unlabeled	Total
In vitro	C^{14}	30	1.5×10^8	1.7×10^8	3.2×10^8
In vitro	C^{14}	10.8	1.9×10^8	9.1×10^{10}	1.9×10^{10}
In vitro	C^{14}	6.9	1.4×10^8	1.2×10^{10}	2.6×10^8
In vitro	C^{14}	30	7.4×10^7	8.6×10^7	1.6×10^8
In vitro	C^{14}	30	3.7×10^7	4.2×10^7	7.9×10^7
In vivo	C^{14}	30	1.4×10^6	1.6×10^6	3.0×10^6
In vivo	H^3	6.7	9.7×10^5	2.9×10^6	3.9×10^6

III. RESULTS

Table II shows the change in the incorporation of thymidine into the DNA of rat spleens with the change in pool size in control and irradiated rats. Figure 1 is a graph of the data given in table II and also contains the data of Stewart et al. for comparison.

The qualitative similarity of our data to that of Stewart et al. (4) is evident. The absolute values are considerably different, but a different system of assay, as well as a different tissue and species, was used. The relationship of the irradiated to the nonirradiated curve is, however, identical.

Over a number of orders of magnitude, the difference between the curve for the irradiated and the nonirradiated animals consists of a shift of the curve to the right. Such a shift could occur simply from an increase in the size of the endogenous pool of thymidine triphosphate. At very large values of pool size, the curves appear to flatten out and also to converge. At these large pool sizes, the use of the tracer method begins to be limiting because the total number of labeled molecules incorporated is very small, and, therefore, the count rate is very low. The low count rate limits the resolution of the system.

IV. DISCUSSION

Most investigators have expressed reservations at one time or another about the possibility that the decrease in specific activity of DNA found after irradiation is due to an increase in the thymidine triphosphate pool size.

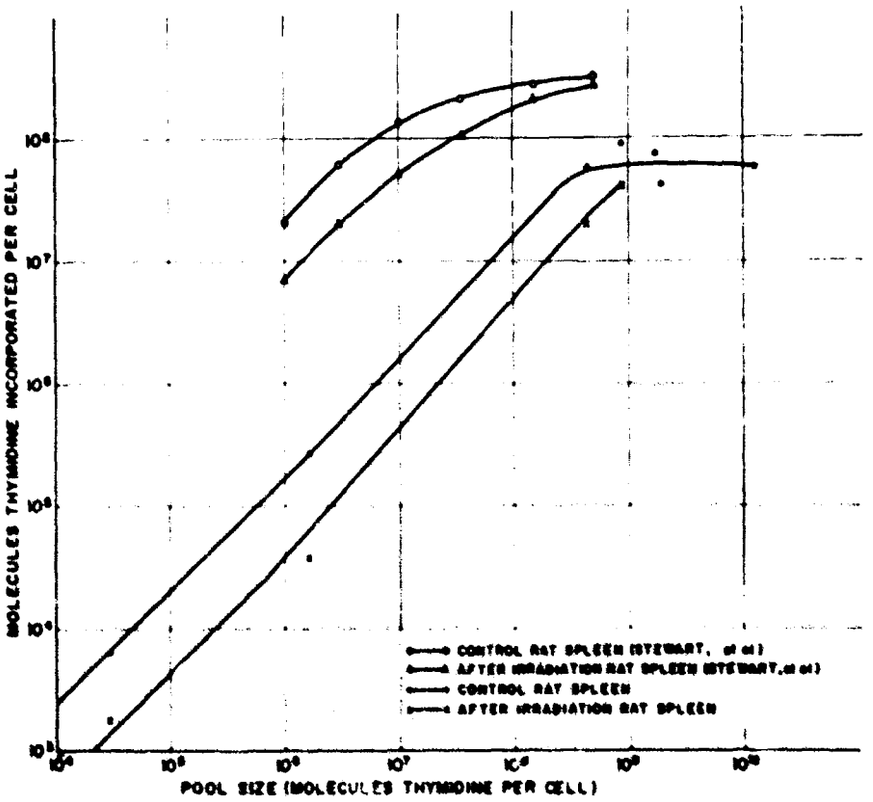

FIGURE 1

Plot of molecules thymidine incorporated versus thymidine pool size.

TABLE II

Incorporation of thymidine in relation to pool size

Total pool size (molecules/cell)	Thymidine incorporated (molecules/cell)	Thymidine incorporated after irradiation (molecules/cell)
1.1×10^{10}	1.3×10^{8}	*
3.2×10^{9}	5.6×10^{7}	*
2.6×10^{9}	5.2×10^{7}	*
1.6×10^{9}	8.4×10^{7}	3.9×10^{7}
7.9×10^{8}	5.3×10^{7}	1.8×10^{7}
3.0×10^{6}	2.6×10^{5}	3.8×10^{4}
3.0×10^{4}	6.6×10^{3}	1.8×10^{3}

*Experiments after irradiation not done because of low count rates.

Huntley and Lajtha (2) studied the effect of pool size on the incorporation of labeled thymidine into mouse bone ma row cells by using thymidine 2-C^{14} of high and low specific activity. They found no significant changes in the percent incorporation into DNA using two different levels of specific activity; however, they covered only a narrow range of total pool size. We have used their data to calculate the pool size according to the method of Stewart et al. (4), and then, using the curves which we obtained, calculated the percent depression expected after irradiation at that pool size. These data are given along with the data of Huntley and Lajtha in table III. In general, their data are in agreement with that predicted by the model.

Stewart et al. (4) used the autoradiographic method to measure thymidine incorporation into DNA. We used a technic based on the extraction of DNA and the measurement of specific activity by chemical analysis and liquid scintillation counting. We were able to get larger pool sizes, but our levels of incorporation are much lower. We have confirmed the relationship which they found of the irradiated and nonirradiated curves of pool size versus incorporation. This relationship suggests that the change in thymidine uptake by DNA after irradiation is due to pool dilution.

Nygaard and Potter (1) studied the conversion of thymidine into the thymine nucleotides after irradiation. Using a pool dilution technic, they found only a slight increase in the thymidine triphosphate pool. Because of this, they concluded that there was no significant effect of pool size on incorporation of thymidine into DNA.

However, further work on the thymidine pool size by using a direct technic is necessary before these data can be accepted without reservation.

TABLE III

Comparative studies on effect of pool size on incorporation of thymidine

Assay system	Isotope	Microcuries	Specific activity (μc./mM.)	Total pool size (molecules/cell)	Percent depression predicted by model	Percent depression found by Huntley and Lajtha (2)
In vitro	H^3	10	0.1	6.02×10^{8}	33	33
In vivo	H^3	10	1.28	4.8×10^{6}	36	36
In vivo	H^3	8	0.36	1.33×10^{6}	32	35
In vitro	H^3	4	0.36	1.0×10^{6}	30	28
In vitro	C^{14}	0.5	0.016	1.87×10^{6}	25	32
In vitro	I^{3}	2	1.9	6.7×10^{6}	24	30

There has been a continuing skepticism about the validity of data purporting to measure rates of DNA synthesis. Our data support the contention of Stewart et al. (4) that the effect of radiation on the rate of DNA synthesis may be an artifact. Pool dilution may occur as a result of depolymerization of DNA, increased catabolism, or even as a result of the repair of damage to DNA molecules. Change in the rate of synthesis per se may not be important in the radiation lesion.

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